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Inflammatory signals increase Fas ligand expression by inner ear cells

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Abstract

There is considerable evidence that hearing and vestibular function can be influenced by immune processes. The inner ear has evolved mechanisms, such as the blood–labyrinthine barrier that limit immune responses and autoimmune processes to reduce the potential for damage to cochlear cells. Recently, expression of Fas ligand (FasL) in some non-lymphoid tissue, as in the anterior chamber of the eye, has been hypothesized to play a role in protection of sensitive organs from activated T-cells. We show that under resting conditions, cochlear cells express little or no FasL. However, after exposure to interferon-γ in vitro, FasL is induced in many neonatal cochlear cells. In addition, we show that FasL is upregulated in adult cochlear cells after induction of a sterile labyrinthitis in vivo. The induction of FasL by inflammation may serve to limit cochlear immune responses, and to protect sensorineural tissue from immune and autoimmune damage.

Keywords: Cochlea; FasL; Immune privilege; Inner ear; Mice

1. Introduction

The FasL–Fas cell-to-cell signaling system plays a crucial role in a variety of biological processes, including development and tissue homeostasis, through the induction of programmed cell death. This system also plays a specific role in immune cytotoxicity, immune cell homeostasis and autoimmunity (Siegel et al., 2000). FasL is a trimeric type II membrane protein of approximately 37 kDa and belongs to the TNF superfamily (Suda and Nagata, 1994; Suda et al., 1993; Takahashi et al., 1994). Its receptor, Fas is a 319-amino acid type I transmembrane glycoprotein with an 80-amino acid region, the death domain, that serves as a module for protein–protein interaction and is critical for apoptosis signaling (Itoh et al., 1991; Tartaglia et al., 1993). When FasL binds to Fas on target cells, caspases are activated and the cells die by programmed cell death (Nagata and Golstein, 1995; Suda et al., 1995).

The Fas death receptor is expressed by many cell types, including most lymphocytes and inflammatory cells. However, as one would expect, expression of FasL is tightly regulated. FasL is expressed strongly on activated T-cells and NK cells, where it participates in killing of infected or neoplastic cells, and in the deletion of autoreactive T-cells (Suda et al., 1995; Bossi and Griffiths, 1999). However, recently a growing number of non-lymphoid cells have been shown to express FasL. For example, cells in the anterior chamber of the eye (Griffith et al., 1995) and Sertoli cells of the testis (Bellgrau et al., 1995) constitutively express FasL. The FasL–Fas system plays an essential role in the eye, as mice which express a nonfunctional Fas receptor display ocular inflammation with inflammatory infiltrates in the choroid and sclera (Jabs and Prendergast, 1991; Jabs et al., 1996). Constitutive FasL expression has been suggested to be involved in protection of immune-privileged sites from immune-mediated damage (Griffith et al., 1995; Griffith et al., 1996; Green and Ferguson, 2001).

There is considerable evidence that hearing and vestibular function can be influenced by immune and autoimmune processes (Ma et al., 2000; Rahman et al., 2001; Ryan et al., 2001). Although the immune system can protect the inner ear, immune-related inflammatory responses can also be

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deleterious, resulting in degeneration of cochlear cells. The inner ear appears to have evolved mechanisms, such as the blood–labyrinthine barrier, which limit immune responses and minimize autoimmunity, in order to prevent damage to the sensitive sensory cells and neurons. Mice deficient in Fas exhibit hearing loss that has been attributed to autoimmune processes (Trune et al., 1989; Ruckenstein et al., 1999). Therefore, it is possible, that FasL participates in the regulation of immunity in the inner ear. The purpose of the present study was to determine whether FasL is expressed in normal cochlear cells and whether there is an upregulation of FasL during cochlear inflammation.

2. Materials and methods

2.1. Animals and dissection

All procedures were approved by an animal subject committee (VA Medical Center, La Jolla, CA, USA) in accordance with the NIH guidelines regarding experimental procedures. Postnatal days 3–5 CBA mice were sacrificed and dissected as described previously (Van de Water and Ruben, 1971; Aletsee et al., 2001). Briefly, after anaesthesia, the skull was opened midsagitally under sterile conditions, and the temporal bone identified after removal of the brain. Employing a dissection microscope, the membranous labyrinth was exposed after gentle removal of the bony and cartilaginous cochlear capsule. The lateral wall tissue, the organ of Corti, the spiral ganglion, and the modiolus including the vasculature and the distal portion of the VIII nerve were microdissected.

2.2. Cell culture

Each of the tissue samples was immediately transferred into the well of a 48-well tissue culture plate (Costar) in Dulbecco's phoshate-buffered saline (Gibco). Four explants were pooled per well. In order to obtain a single cell suspension, explants were digested with 1 mg/ml trypsin, 10 mg/ml collagenase, and 1 mg/ml elastase (Sigma, St. Louis, USA) for 40 min at 37 °C. After removal of the supernatant, 500 μl PBS containing 5% fetal calf serum (FCS), Hepes buffer 0.025 M, and penicillin 30 U/ml was added per well. Cell dispersion was accomplished by trituration using a 1-cm^3 syringe. Spleen tissue served as a control and was treated identically. Each respective cell population was split into two. One aliquot was stimulated with 100 IU/ml recombinant mouse interferon-γ (R&D Systems, USA) for 24 h, which was repeated for a total of 48 h of interferon stimulation. The other aliquot remained unstimulated. Interferon-γ was employed to stimulate the inner ear cells in culture, because this cytokine is an early mediator of inflammation and has been shown to be a potent inducer of FasL in several cellular systems (Choi et al., 1999). For the time course study, the cells were harvested after 6, 12, 24, and 48 h of interferon stimulation (for 6–24 h, $n=1$; for 48 h, $n=5$). All tissues were cultured at 37 °C and 5% CO_2.

2.3. Fluorescent staining and flow cytometric analysis (FACS)

After final removal of the supernatant, incubation with a purified rat IgG anti-mouse CD16/CD32 monoclonal antibody was performed for 10 min at 4 °C (2 μl/well) to block nonspecific adherence of antibodies to mouse Fc receptors (Fc Block, Pharmingen). Cells were then washed and incubated for 30 min at 37 °C with a polyclonal goat anti-mouse FasL antibody (1:100, Santa Cruz Biotechnology, USA). The cells were washed twice with PBS and incubated for 45 min at 4 °C with a rabbit anti-goat IgG FITC-conjugated antibody (1:100, Jackson Immuno, USA). Cells were washed twice with cold PBS, gently detached with 400 μl accutase (Cell Technology, San Diego, CA, USA) and gently removed from the wells. Flow cytometric acquisition of the samples was performed on a FACScan (Becton Dickinson, San Jose, CA) using a standard Cell Quest acquisition/analysis software. Five thousand live events, which were gated on their forward/side scatter characteristics, were collected. Unstimulated spleen cells were used as a negative control for nonspecific binding of the secondary antibody. The primary antibody was replaced by the same volume of PBS and the threshold was adjusted so that less than 1% of the cells were gated. This threshold was then used for all samples. As an additional control, each inner ear cell population was individually stimulated and unstimulated with interferon-γ for 48 h and analyzed using the secondary antibody only.

2.4. Induction of sterile labyrinthitis in mice

This procedure has been described in detail in a previous publication (Takahashi and Harris, 1988). Briefly, mice were immunized subcutaneously with the antigen keyhole limpet hemocyanin (KLH), (Pacific Biomarine Supply, Venice, CA) in complete Freunds adjuvant and boosted 10 days later with KLH in incomplete Freunds adjuvant. For the inner ear challenge, the right middle ear bulla was opened and the posterior half of the tympanic membrane removed. A microhole was drilled into the basal turn of the cochlea and 5 μl KLH injected slowly into the cochlea. The hole was sealed with bone wax. Seven days after the inner ear challenge, the animals were deeply anaesthetized and perfused with intracardiac perfusion with periodate–lysin–paraformaldehyde fixative. After removal and decalcification of the temporal bones with 4% EDTA at 4 °C, the tissue was frozen in OCT compound (Tissue Tec, Naperville, IL, USA).

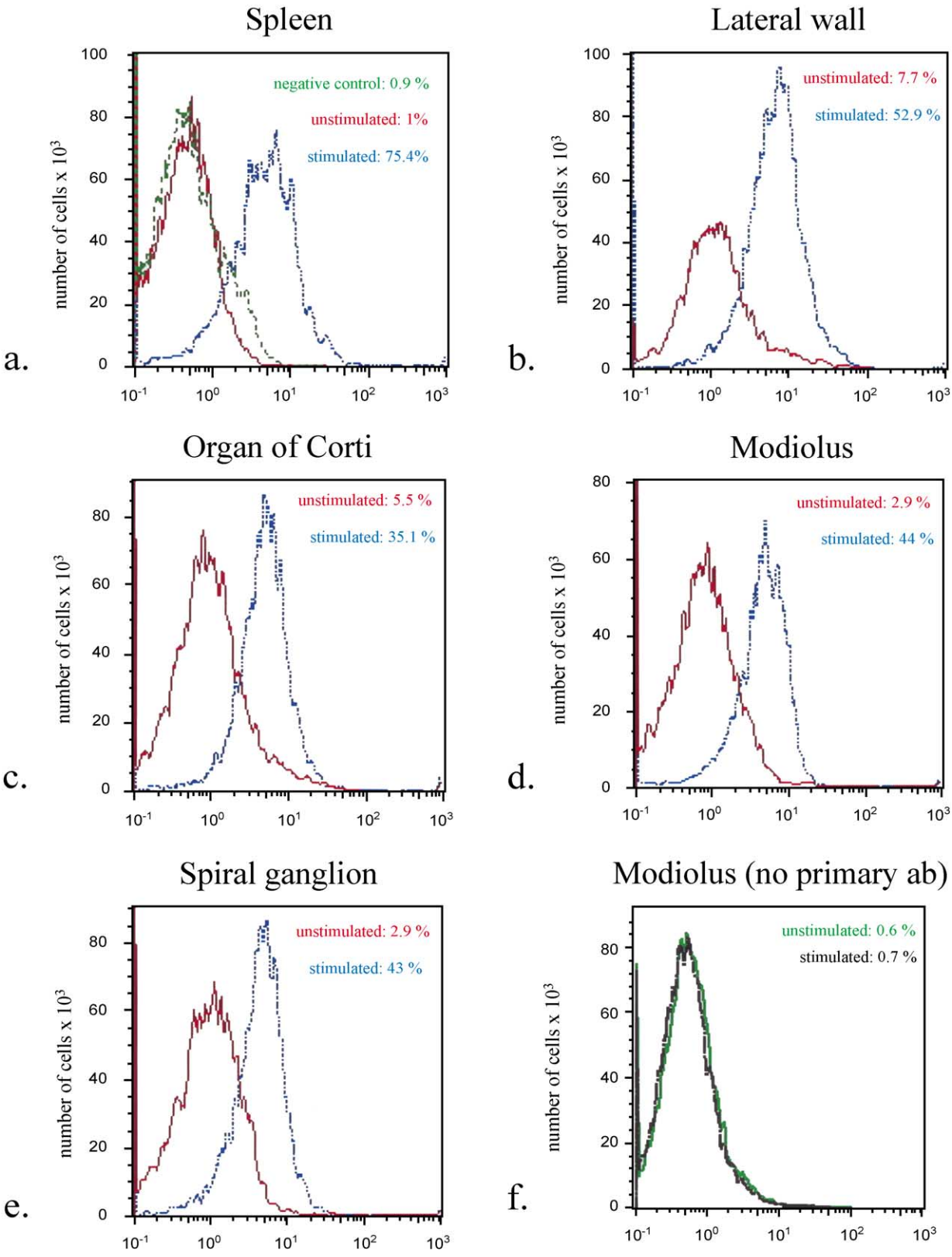

Fig. 1. Spleen, lateral wall, organ of Corti, modiolus, and spiral ganglion were harvested and dissociated, and half of the cells were treated with interferon-γ for 48 h. Both populations were then subjected to FACS analysis to measure FasL expression. A representative run is shown. The green line in a denotes sorting of spleen cells without the FasL antibody, as a negative control. The red and blue lines represent sorting of untreated or interferon-γ-treated cells, respectively, using both the primary and secondary antibodies. The green and black line in f denote sorting of untreated or interferon-γ-treated cells, respectively, using the secondary antibody only. No primary ab = no primary antibody.

2.5. Immunohistochemistry

The frozen temporal bone was serially sectioned (5–7 μm) in the direction of the modiolus. After air-drying of the sections, an indirect immunoperoxidase technique was used with a primary polyclonal goat anti-mouse FasL antibody (1:75 to 1:200, Santa Cruz Biotechnology) incubated for 1 h at room temperature. Further staining was carried out employing a secondary rabbit anti-goat IgG antibody (1:2000, Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA) and the color reaction was performed with DAB (Vector Laboratories). A slight counterstaining with hematoxylin was performed at the end. For negative control, the primary antibody was replaced by PBS or goat serum. Murine spleen was processed in the same way and served as positive control tissue.

3. Results

3.1. Cell culture

The number of inner ear cells ranged from 8000 to 12 000 per well. The survival rate was about 65–75% after 48 h in culture, as shown by trypan blue exclusion, irrespective of the population and interferon-γ stimulation.

3.2. FACS results with and without interferon-γ stimulation

FasL was expressed in unstimulated spleen cultures, in a resting state, at a value of 1% with an increase to 75% after interferon-γ treatment (Fig. 1a). On resting inner ear cells, the ligand was expressed at levels ranging from 3% to 8% (Fig. 1b–e). After stimulation with interferon-γ, FasL was

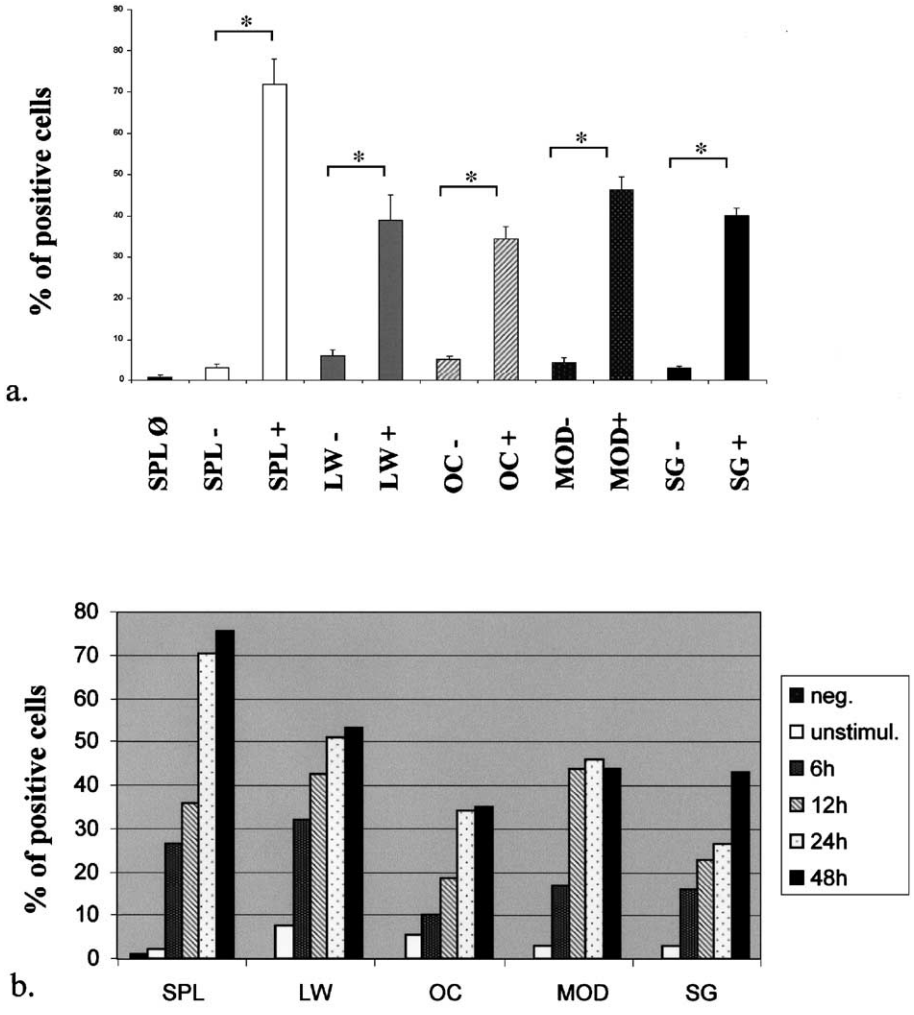

Fig. 2. (a) Represents the average of five independent runs of FACS after 48 h interferon-γ stimulation, as illustrated in Fig. 1. Statistical analysis was performed using the Mann–Whitney *U*-test. Bars represent mean values, standard errors are indicated by lines. SPL Ø = negative control (unstimulated spleen without FasL antibody), SPL – = unstimulated spleen, SPL + = stimulated spleen, LW – = unstimulated lateral wall, LW + = stimulated lateral wall, OC – = unstimulated organ of Corti, OC + = stimulated organ of Corti, MOD – = unstimulated modiolus, MOD + = stimulated modiolus, SG – = unstimulated ated spiral ganglion, SG + = stimulated spiral ganglion ($*p < 0.01$; in each group, the unstimulated value was compared to the stimulated value). (b) Represents a time course study with FACS runs after 0, 6, 12, 24, and 48 h of interferon-γ stimulation, as illustrated in Fig. 1. Each bar represents one FACS run.

upregulated 48 h later to values from 35% to 53% (Figs. 1b–e and 2a). In the control experiment, sorting of unstimulated and stimulated inner ear cells without the primary antibody resulted in lower than 2% positivity for all inner ear portions, as an example, the result for sorting of cells from the modiolus is shown (Fig. 1f). In the time course study, we observed an increase of FasL expression over time, with the maximal expression at 24 h after the initial stimulation with interferon-γ in spleen, lateral wall, organ of Corti, and modiolus. Maximal expression was observed 48 h after the initial stimulation with interferon-γ in spiral ganglion (Fig. 2b).

3.3. FasL expression during labyrinthitis

Normal spleen tissue displayed strong immunoreactivity for FasL in the interfollicular region (Fig. 3a). Except for a faint staining of the spiral ganglion, no FasL immunoreac-

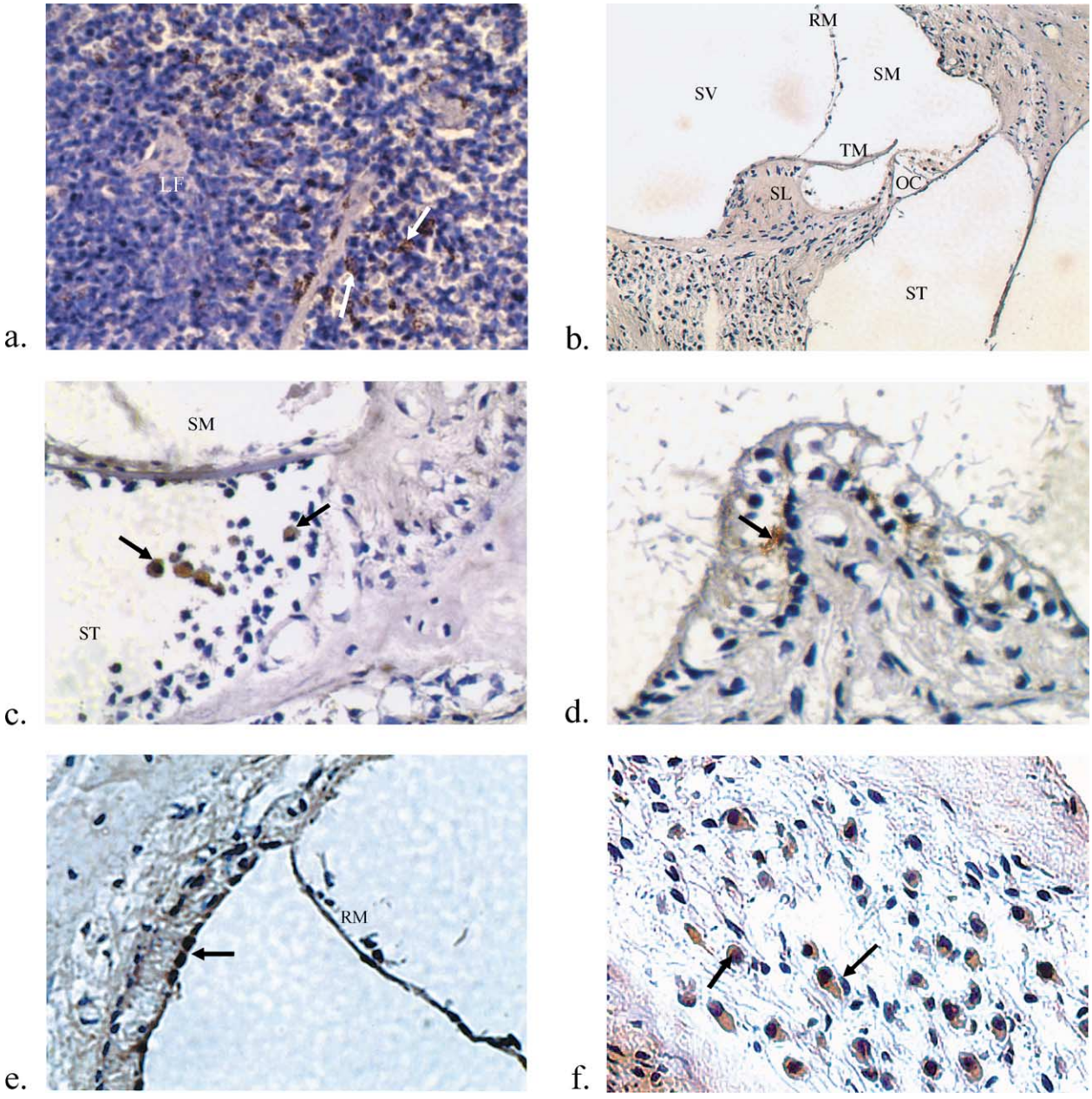

Fig. 3. Immunohistochemical localization of FasL molecules in normal spleen (a), normal inner ear (b), and inner ear during labyrinthitis (c–f). (a) Normal spleen, arrows indicate FasL-positive cells (brown) in the interfollicular region. LF = lymphoid follicle. (b) Normal cochlea demonstrating only background FasL labeling. OC = organ of Corti, RM = Reissner membrane, SL = spiral limbus, SM = scala media, ST = scala tympani, SV = scala vestibuli, TM = tectorial membrane. (c) Strongly FasL-positive inflammatory cells (arrows) in the scala tympani 7 days after induction of labyrinthitis. (d) Moderately FasL-positive supporting cells (arrow) of the crista ampullaris in labyrinthitis. (e) Moderately FasL-positive marginal cells (arrow) of the stria vascularis in labyrinthitis. (f) Moderately FasL-positive spiral ganglion neurons (arrows) in labyrinthitis.

tivity was detected in the untreated inner ear (Fig. 3b). One week after induction of a sterile labyrinthitis, the treated inner ear showed a strong FasL immunoreactivity in ~ 20% of the infiltrating leukocytes (Fig. 3c). Supporting cells of the crista ampullaris, marginal cells of the stria vascularis and neurons of the spiral ganglion showed moderate FasL immunostaining in the treated inner (Fig. 3d–f), whereas the organ of Corti displayed a weak FasL immunostaining in supporting cells (data not shown). When the primary antibody was replaced with PBS or goat serum, background staining was very low in all sections (data not shown).

4. Discussion

FACS is an established method in experimental biology in a variety of fields, including brain and immunology research (Weller et al., 1996; Brehm et al., 1999; Herzenberg and De Rosa, 2000). However, its application to the study of the inner ear is new. This technique offers advantages, such as preservation of antigenicity in unfixed tissue and reproducible stimulation of cells in culture. Disadvantages of FACS include trauma associated with the dissociation process, changes in cellular characteristics due to culture conditions, and heterogeneity of cell populations. For these reasons, we compared our FACS findings to immunohistochemical data using an established model of inner ear inflammation in the adult mouse (Harris and Ryan, 1984).

When dispersed neonatal cochlear cells from lateral wall, organ of Corti, modiolus and spiral ganglion were stimulated with repeated doses of interferon-γ, a significant increase of FasL was detected using FACS, in contrast to untreated control cells. In immune-mediated labyrinthitis, FasL was detected in infiltrating inflammatory cells and in subpopulations of cochlear cells such as spiral ganglion neurons, supporting cells in sensory epithelia, and marginal cells of the stria vascularis. Agreement of the results obtained with the two methods is therefore good, but it was not complete. For example, we saw little FasL immunolabeling in modiolar tissue sections, but FACS analysis of interferon-γ-treated modiolar samples showed many cells that were positive for FasL. This may be related to differences in the nature of the two methods. The FACS data were obtained during 6–48 h of inflammatory stimulation with a single cytokine, while the histochemical analysis was obtained after 1 week of naturally mediated inflammation. In any event, the data from both methods support the expression of FasL by the inflamed inner ear.

The expression of FasL in the inner ear implies a functional role for FasL–Fas signaling. Recently, it has been shown that treatment of cultured cochlear sensory cells with cisplatin results in increased expression of Fas receptor (Van de Water et al., 2001). In addition, the MRL-Fas$^{lpr/lpr}$ mouse, which expresses a nonfunctional Fas receptor, displays progressive hearing loss not seen in the background MRL strain. This may be related to autoimmunity since this mouse shows progressive accumulation of autoreactive T-lymphocytes. However, the most striking inner ear pathology of the Fas mutant mouse is found in the stria vascularis, with progressive hydrops and degeneration of strial cells in the absence of leukocyte infiltration of the stria (Trune et al., 1989; Ruckenstein et al., 1999). These data provide evidence that the Fas–FasL system plays a role in the inner ear, but the nature of this role remains obscure.

The central nervous system (CNS), and the inner ear by extension, were originally thought to be immunoprivileged sites through the actions of the blood–brain and blood–labyrinthine barriers, respectively. However, this view has been altered by many observations. With respect to the inner ear, systemic immunity has been shown to protect the cochlea from subsequent viral labyrinthitis (Woolf et al., 1985). Antigens introduced into the cochlea of a naïve animal produce a brisk systemic immune response (Harris, 1983; Harris et al., 1985), while introduction of antigens into the inner ear of systemically immunized animals results in hearing loss due to a vigourous secondary immune response (Woolf and Harris, 1986; Ma et al., 2000). The concept that autoimmunity might damage the labyrinth resulting in bilateral, progressive sensorineural hearing loss (SNHL) was introduced in McCabe (1979). Autoimmunity has since been proposed as an etiology for Meniere's disease (Hughes et al., 1988), sudden SNHL (Moskowitz et al., 1984), and acute vertigo (Hughes et al., 1985). These and other data provide evidence that immune mechanisms and their associated inflammatory response can cause damage to sensitive auditory and/or vestibulary cells.

While the inner ear does not appear to be immunoprivileged, the blood–labyrinthine barrier does exclude lymphocytes and most immunoglobulins from the normal cochlea. The inner ear may have evolved additional mechanisms by which to limit immune damage. In the corneal epithelium, iris, and ciliary cells of the eye, as well as in Sertoli cells of the testis, FasL is constitutively expressed (Bellgrau et al., 1995; Griffith et al., 1995). It has been proposed that activated lymphocytes that enter these sites are killed by the FasL expressed on these cells (Griffith et al., 1996; Bellgrau et al., 1995). However, in contrast to the eye and the testis, we find little or no constitutive expression of FasL in the inner ear. Only upon stimulation with interferon-γ or after the induction of labyrinthitis did we observe FasL expression on subpopulations of cochlear cells such as spiral ganglion neurons, supporting cells in sensory epithelia, and marginal cells of the stria vascularis. This is consistent with the idea that the inner ear is not an immunologically privileged site. Rather, the inner ear seems to display restricted immunity unless provoked. It is possible that the upregulation of FasL limits immune responses in the inner ear, and so protects the tissues of the labyrinth from the later stages of immune-mediated damage. The advantages of active immunity may outweigh the advantages of immune privilege in the inner ear, until inflammation is strongly

activated. If this interpretation is correct, it is possible that application of FasL might protect the inner ear from immune-mediated damage.

A striking feature of the results is the fact that all of the cell populations tested by FACS analysis showed increases in FasL. This may reflect the fact that many of the cells of the cochlea are exposed to cochlear fluids, and thus are vulnerable to damage from leukocytes that are typically observed in cochlear fluids during inflammation. It should also be noted that each population tested consisted of several different inner ear cell types. Thus, there were a number of potential candidates for FasL expression in each. Indeed, the FACS data indicated that only about 40% of the cells in each population were positive for FasL immunoreactivity. Similarly, the histochemical data indicated that only subsets of the cells in different regions of the cochlea were labeled with anti-FasL antibodies.

The specific cell types labeled in the cytochemical study may reflect potential cellular targets of FasL and/or the cochlear cells meant to be protected by FasL expression. Production of FasL by infiltrating leukocytes may target other inflammatory cells, in order to limit inflammatory damage to the inner ear. Expression by supporting cells in inner ear sensory epithelia may be designed to protect the delicate sensory cells. It can be argued that the receptor cells themselves are so specialized that they may be unable to express FasL. In the stria vascularis, the marginal cells may be protecting themselves and underlying cells from inflammatory cells in the endolymph, since they directly face this fluid compartment.

While FasL expression was seen in many inner ear cells types, the time course of expression observed by FACS differed between cell populations of different origins. The most rapid response was observed in the modiolar samples, for which FasL positivity reached maximal levels in 12 h, while the slowest response was seen in the spiral ganglion, which did not peak until 48 h. The rapid modiolar response may occur because this is the normal site of leukocyte entry into the inner ear. The spiral modiolar vein has been shown to be the most active site of inner ear leukocyte recruitment during inflammation (Harris et al., 1990). Cells in the modiolus may thus be programmed to respond more rapidly to inflammatory stimuli. The delayed response seen in the spiral ganglion may reflect the fact that spiral ganglion neuron cell bodies are completely sheated in myelin. The cytochemical results indicated that the neurons expressed FasL, not the glial cells or fibroblasts of the ganglion. Access of interferon-γ to the neurons may have been impeded by shielding effects of myelin, thus delaying the response.

It should be noted that FasL expression in the inflamed inner ear may have functions unrelated to protection from immune and inflammatory cells. For example, it has recently been demonstrated that FasL inhibits angiogenesis beneath the retina (Kaplan et al., 1999). The formation of new blood vessels could be deleterious for the inner ear and, in fact, angiogenesis is not generally observed in inflamed cochleas. The induced expression of FasL could help to limit neovascularization that might otherwise be stimulated by inflammatory mediators.

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